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(54) **LACTIC ACID BACTERIAL CULTURE**

MILCHSÄUREBAKTERIENKULTUR

CULTURE DE BACTÉRIES LACTIQUES

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(73) Proprietor: **Chr. Hansen A/S**
2970 Hoersholm (DK)

(72) Inventor: **JANNIK TORBEN, Vindeloev**
DK-1905 Frederiksberg C (DK)

(74) Representative: **Hoffmann Eitle**
Patent- und Rechtsanwälte PartmbB
Arabellastraße 30
81925 München (DE)

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- **BARRETTE J ET AL: "The production of mixed cultures containing strains of Lactococcus lactis, Leuconostoc cremoris and Lactobacillus rhamnosus, on commercial starter media", JOURNAL OF INDUSTRIAL MICROBIOLOGY AND BIOTECHNOLOGY, BASINGSTOKE, GB, vol. 25, no. 6, 1 December 2000 (2000-12-01), pages 288-297, XP002463787, ISSN: 1367-5435, DOI: 10.1038/SJ.JIM.7000105**
- **BARRETTE et al.: "The production of mixed cultures containing strains of Lactococcus lactis, Leuconostoc cremoris and Lactobacillus rhamnosus, on commercial starter media", Journal of Industrial Microbiology and Biotechnology, vol. 25, no. 6, 2000, pages 288-297,**

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Description

FIELD OF INVENTION

[0001] The present invention relates to a bacterial starter culture useable in a method for preparing a camembert type cheese with a direct vat set starter culture.

BACKGROUND OF INVENTION

[0002] By concentrating the milk solids, lowering pH with lactic acid, removing sugar, and adding salt, cheese was traditionally a way to conserve milk for later consumption. Today, cheese is mainly appreciated for its organoleptic properties.

[0003] Conventional cheese making consist of three key steps: Coagulation (formation of a casein gel also called the curd), Drainage (the expulsion of whey from the curd), and Maturation (ripening of the curd into cheese).

[0004] During these steps, coagulants (such as rennet) are used to coagulate the milk, and starter cultures are used to lower pH and reduce sugar levels by transforming the milk lactose to lactic acid by fermentation, and provide enzymes for cheese ripening.

[0005] In camembert, it is well known that the balance between the rate of acidification and the rate of whey expulsion is essential to obtain the right cheese quality. Even slight imbalances will impact the texture, flavour and taste of the cheese. For a comprehensive review of the effect of acidification during coagulation, drainage, on cheese quality and ripening see B. Mieton et al.

[0006] Traditionally, camembert type cheeses are produced with undefined bulk starter cultures (starters of unknown strain composition, prepared locally at the dairy). The use of undefined bulk starter cultures has several drawbacks, e.g. lack of consistency and batch-to-batch variation, and the undefined starters may contain strains with undesired properties, such as resistance to antibiotics, pro-phages and production of allergens.

[0007] Several attempts have been made to replace the undefined bulk starters with well-defined cultures, especially DVS cultures, but without any commercial success, apparently because it has turned out to be impossible to provide a well-defined starter culture that is able to mimic the properties of the undefined starter cultures hitherto used (eg with respect to acidification curve, acidification time, and texture, flavour (eg bitterness) of the resulting cheese, etc).

[0008] BARRETTE J ET AL, JOURNAL OF INDUSTRIAL MICROBIOLOGY AND BIOTECHNOLOGY, BASINGSTOKE, GB, vol. 25, no. 6 (2000-12-01), pages 288-297, XP002463787, ISSN: 1367-5435, DOI: 10.1038/SJ.JIM.7000105 discloses mixed starter cultures comprising *L lactis* for use in cheese making.

[0009] Thus, there is still a need for a well-defined starter culture that can be used for the production of camembert type cheese.

SUMMARY OF INVENTION

[0010] It has surprisingly turned out that it is possible to provide a well-defined starter culture that is able to mimic the bulk starter cultures traditionally used, especially with respect to the specific acidification curve that is crucial for the production of camembert cheese.

[0011] The present inventor has revealed that the well-defined culture should contain several strains with different acidification characteristics. Surprisingly, the present inventor has found out that the starter culture should comprise - besides a bacterial culture that normally is found in a starter culture - a bacteria culture that is not able to grow in milk added 4% salt.

[0012] In accordance with the above surprising findings, there is described a method for preparing a cheese, preferably a soft cheese (e.g. a cheese of the camembert type) comprising the steps:

Adding to a milk substrate

[0013]

a1) a bacteria culture of type M (a lactic acid bacteria culture that has a maximum acidification rate of more than or equal to 0.05 pH units per hour but less than 0.15 pH units per hour in B-milk at 30 °C);
a2) a bacteria culture of type R (a lactic acid bacteria culture that has a maximum acidification rate of more than or equal to 0.40 pH units per hour in B-milk at 30 °C, and a maximum acidification rate of less than 0.05 pH units per hour in B-milk added 4% salt at 30 °C);
a3) optionally a bacteria culture of type S (a lactic acid bacteria culture that has a maximum acidification rate of more than or equal to 0.40 pH units per hour in B-milk at 30 °C and has a maximum acidification rate of more than 0.05 pH units per hour in B-milk added 4% salt at 30 °C); and
a4) a coagulant.

[0014] The present invention relates to a lactic acid bacterial (LAB) culture comprising

A1) a bacterial culture of type M (a lactic acid bacteria culture that has a maximum acidification rate of more than or equal to 0.05 pH units per hour but less than 0.15 pH units per hour in B-milk at 30 °C)
A2) a bacterial culture of type R (a lactic acid bacteria culture that has a maximum acidification rate of more than or equal to 0.40 pH units per hour in B-milk at 30 °C, and has a maximum acidification rate of less than 0.05 pH units per hour in B-milk added 4% salt at 30 °C); and
A3) optionally a bacterial culture of type S (a lactic acid bacteria culture that has a maximum acidification rate of more than or equal to 0.40 pH units per hour in B-milk at 30 °C and has a maximum acidifi-

cation rate of more than 0.05 pH units per hour in B-milk added 4% salt at 30 °C); and wherein the culture of type M comprises bacteria belonging to a strain selected from the group consisting of: *L. lactis* ssp. *cremoris* CHCC11552 (DSM 26566); *L. lactis* ssp. *cremoris* CHCC11583 (DSM 26567); *L. lactis* ssp. *cremoris* CHCC11603 (DSM 26568); *L. lactis* ssp. *cremoris* CHCC12085 (DSM 26569); and *L. lactis* ssp. *cremoris* CHCC13347 (DSM 26570); and mutants thereof, wherein the culture of type R comprises bacteria belonging to a strain selected from the group consisting of: *L. lactis* ssp. *lactis* CHCC10778 (DSM 26564); and *L. lactis* ssp. *lactis* CHCC11277 (DSM 26565); and mutants thereof and wherein the culture of type S comprises bacteria belonging to a strain selected from the group consisting of: *L. lactis* ssp. *cremoris* CHCC4421 (DSM 26561); and *L. lactis* ssp. *lactis* CHCC9673 (DSM 26563); and mutants thereof.

[0015] There is also described a cheese obtainable by the method described above.

DETAILED DISCLOSURE

[0016] There is described a method for preparing a soft cheese (e.g. cheese of the camembert type) comprising the steps:

a) Adding to a milk substrate

a1) a bacteria culture of type M (a lactic acid bacteria culture that has a maximum acidification rate of more than or equal to 0.05 pH units per hour but less than 0.15 pH units per hour in B-milk at 30 °C);
a2) a bacteria culture of type R (a lactic acid bacteria culture that has a maximum acidification rate of more than or equal to 0.40 pH units per hour in B-milk at 30 °C, and a maximum acidification rate of less than 0.05 pH units per hour in B-milk added 4% salt at 30 °C);
a3) optionally a bacteria culture of type S (a lactic acid bacteria culture that has a maximum acidification rate of more than or equal to 0.40 pH units per hour in B-milk at 30 °C and has a maximum acidification rate of more than 0.05 pH units per hour in B-milk added 4% salt at 30 °C); and
a4) a coagulant.

[0017] Preferred embodiments of this aspect are:

- A method comprising the steps:

a) Adding to a milk substrate

a1) a bacteria culture of type M; and
a2) a bacteria culture of type R; and
a3) a bacteria culture of type S; and
a4) a coagulant; and

b) Separating the curd from the whey; and
c) Adding a mold culture to the curd.

- A method of this aspect, wherein the culture in a1) is added at a concentration in the range from 10E4 to 10E10 cell forming units (CFU) per liter milk substrate.
- A method of this aspect, wherein the culture in a2) is added at a concentration in the range from 10E4 to 10E10 cell forming units (CFU) per liter milk substrate.
- A method of this aspect, wherein the culture in a3) is added at a concentration in the range from 10E4 to 10E10 cell forming units (CFU) per liter milk substrate.
- A method of this aspect, wherein a least one of the cultures of types M, R and S is added in liquid, frozen or freeze-dried form.
- A method of this aspect, wherein at least two of the cultures of types M, R and S are added simultaneously to the milk substrate.
- A method of this aspect, wherein the cultures of types M, R and S (if added) are added in the form of direct vat set cultures.
- A method of this aspect, wherein the cultures of types M, R and S (if added) are added as a single culture, e.g. as a blend packaged in the same package.
- A method of this aspect, wherein the culture of type M comprises one, two, three or more strains.
- A method of this aspect, wherein the culture of type M comprises one, two, three or more strains of the species Lactococcus lactis.
- A method of this aspect, wherein the culture of type R comprises one, two, three or more strains.
- A method of this aspect, wherein the culture of type R comprises one, two, three or more strains of the species Lactococcus lactis.
- A method of this aspect, wherein the culture of type S comprises one, two, three or more strains.
- A method of this aspect, wherein the culture of type S comprises one, two, three or more strains of the species Lactococcus lactis.

- A method of this aspect, wherein the mold culture is selected from the group consisting of Penicillium species, such as Penicillium camemberti or Penicillum candida, Geotrichum species, such as Geotrichum candidum.

- A method of this aspect, wherein the coagulant is selected from the group consisting of: an aspartic protease; a chymosin of animal origin; a fermentation produced chymosin (such as CHY-MAX® (bovine) or CHY-MAX® M (camel)); a fungal protease, etc.

[0018] The invention relates to a lactic acid bacterial (LAB) culture comprising

A1) a bacterial culture of type M (a lactic acid bacteria culture that has a maximum acidification rate of more than or equal to 0.05 pH units per hour but less than 0.15 pH units per hour in B-milk at 30 °C)
A2) a bacterial culture of type R (a lactic acid bacteria culture that has a maximum acidification rate of more than or equal to 0.40 pH units per hour in B-milk at 30 °C, and has a maximum acidification rate of less than 0.05 pH units per hour in B-milk added 4% salt at 30 °C); and
A3) optionally a bacterial culture of type S (a lactic acid bacteria culture that has a maximum acidification rate of more than or equal to 0.40 pH units per hour in B-milk at 30 °C and has a maximum acidification rate of more than 0.05 pH units per hour in B-milk added 4% salt at 30 °C); wherein the culture of type M comprises bacteria belonging to a strain selected from the group consisting of: *L. lactis* ssp. *cremoris* CHCC11552 (DSM 26566); *L. lactis* ssp. *cremoris* CHCC11583 (DSM 26567); *L. lactis* ssp. *cremoris* CHCC11603 (DSM 26568); *L. lactis* ssp. *cremoris* CHCC12085 (DSM 26569); and *L. lactis* ssp. *cremoris* CHCC13347 (DSM 26570); and mutants thereof, wherein the culture of type R comprises bacteria belonging to a strain selected from the group consisting of: *L. lactis* ssp. *lactis* CHCC10778 (DSM 26564); and *L. lactis* ssp. *lactis* CHCC11277 (DSM 26565); and mutants thereof and wherein the culture of type S comprises bacteria belonging to a strain selected from the group consisting of: *L. lactis* ssp. *cremoris* CHCC4421 (DSM 26561); and *L. lactis* ssp. *lactis* CHCC9673 (DSM 26563); and mutants thereof.

[0019] Interesting embodiments are:

- A culture of the invention comprising

  A1) a bacterial culture of type M; and
  A2) a bacterial culture of type R; and
  A3) a bacterial culture of type S.

- A culture of the invention comprising

  A1) 5-90% of the culture of type M; and
  A2) 5-90% of the culture of type R; and
  A3) 0-90% of the culture of type S;

  the ratio is calculated as CFU relative to the total CFU of the culture.

- A culture of the invention comprising

  A1) 5-90% of the culture of type M; and
  A2) 5-90% of the culture of type R; and
  A3) 5-90% of the culture of type S;

  the ratio is calculated as CFU relative to the total CFU of the culture.

- A culture of the invention comprising from 10E8 to 10E12 cell forming units (CFU) of the culture of type M per g culture.

- A culture of the invention comprising from 10E8 to 10E12 cell forming units (CFU) of the culture of type R per g culture.

- A culture of the invention comprising which comprises from 10E8 to 10E12 cell forming units (CFU) of the culture of type S per g culture.

- A culture of the invention, which is in liquid, frozen or freeze-dried form.

- A culture of the invention, wherein the cultures of types M, R and S (if present) is a mixed culture in the same package.

- A culture of the invention, wherein at least one of the cultures of types M, R and S (if present) is packaged as individually, e.g. as a kit-of-parts. Thus, the culture might be packaged and supplied to the customer in one, two, three or more separate packages.

- A culture of the invention, wherein the culture of type M comprises one, two, three or more strains.

- A culture of the invention, wherein the culture of type M comprises one, two, three or more strains of the species Lactococcus lactis.

- A culture of the invention, wherein the culture of type R comprises one, two, three or more strains.

- A culture of the invention, wherein the culture of type R comprises one, two, three or more strains of the species Lactococcus lactis.

- A culture of the invention, wherein the culture in of type S comprises one, two, three or more strains.

- A culture of the invention, wherein the culture of bacteria of type S comprises one, two, three or more strains of the species Lactococcus lactis.
- A culture of the invention, wherein the concentration of bacteria is in the range of 10E9 to 10E13 CFU per g.
- A culture of the invention, which is in frozen form.
- A culture of the invention, which is in freeze dried form.
- A culture of the invention, which is in liquid form.
- A culture of the invention, which is in dried form.

[0020] There is also described a cheese obtainable by the method as described above, and a cheese comprising a LAB culture of the invention. The cheese may be a camembert type cheese.

[0021] There is also described a method for acidifying a milk substrate by adding a culture of the invention to the milk substrate. The product of the process may be a fermented milk product ready for consumption, such as yoghurt, buttermilk, sour cream, etc, or the product may be further processed, e.g. by addition of an enzyme such as a coagulant. Such products are also a part of the present invention.

[0022] In the last aspect, the present invention relates to the use of a culture of the invention a process for producing cheese.

DEFINITIONS

[0023] In the present context, the term "soft cheese" or "camembert type cheese" refers to soft-ripened cheese such as camembert, brie, etc.

[0024] In the present context, the term "cheese" comprises soft cheese and hard or semi hard cheese, for example Cheddar, Red Leicester, American cheese, gouda, edam, emmental, an Italian cheese such as Parmesan, Parmigiano, Regiano, Grana Padano, Provolone, Pecorino, or Romano.

[0025] As used herein, the term "lactic acid bacterium" designates a gram-positive, microaerophilic or anaerobic bacterium, which ferments sugars with the production of acids including lactic acid as the predominantly produced acid, acetic acid and propionic acid. The industrially most useful lactic acid bacteria are found within the order "Lactobacillales" which includes *Lactococcus* spp., *Streptococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pseudoleuconostoc* spp., *Pediococcus* spp., *Brevibacterium* spp., *Enterococcus* spp. and *Propionibacterium* spp.

[0026] In the present context, the term "a culture of type M" (or "a culture of bacteria belonging to the group M") designates a lactic acid bacteria culture that has a maximum acidification rate of more than or equal to 0.05 pH units per hour but less than 0.15 pH units per hour in B-milk.

[0027] In the present context, the term "a culture of type S" (or "a culture of bacteria belonging to the group S") designates a lactic acid bacteria culture that has a maximum acidification rate of more than or equal to 0.40 pH units per hour in B-milk, but will not acidify B-milk added 4% salt (less than 0.05 pH units per hour). In the present context, "salt" is NaCl.

[0028] In the present context, the term "a culture of type R" (or "a culture of bacteria belonging to the group R") designates a lactic acid bacteria culture that has a maximum acidification rate of more than or equal to 0.40 pH units per hour in B-milk and acidify B-milk added 4% salt (i.e. having a maximum acidification rate of more than 0.05 pH units per hour).

[0029] It is understood that in the above definitions the culture is grown in B-milk (9.5% reconstituted skimmed milk heated to 99C for 30 min) at 30 degrees C. The inoculation rate is not important, as the acidification rate is substantially the same when the milk is inoculated with from 10E2 to 10E10 CFU/ml milk. It is however preferred that the inoculation rate is 10E8 CFU/ml milk in the definitions. The term "culture" refers to a pure strain (a single strain) or a co-culture of pure strains, i.e. an ensemble of pure strains grown together. The strains according to the invention are Lactococcus species.

[0030] In the present context, the term "milk substrate" may be any raw and/or processed milk material that can be subjected to fermentation according to the method of the invention. Thus, useful milk substrates include, but are not limited to, solutions/suspensions of any milk or milk like products comprising protein, such as whole or low fat milk, skim milk, buttermilk, reconstituted milk powder, condensed milk, dried milk, whey, whey permeate, lactose, mother liquid from crystallization of lactose, whey protein concentrate, or cream. Obviously, the milk substrate may originate from any mammal, e.g. being substantially pure mammalian milk, or reconstituted milk powder. Preferably, at least part of the protein in the milk substrate is proteins naturally occurring in milk, such as casein or whey protein. However, part of the protein may be proteins which are not naturally occurring in milk. Prior to fermentation, the milk substrate may be homogenized and pasteurized according to methods known in the art. The term "milk" is to be understood as the lacteal secretion obtained by milking any mammal, such as cows, sheep, goats, buffaloes or camels. In a preferred embodiment, the milk is cow's milk. The term milk also comprises soy milk. Optionally the milk is acidified, e.g. by addition of an acid (such as citric, acetic or lactic acid), or mixed, e.g. with water. The milk may be raw or processed, e.g. by filtering, sterilizing, pasteurizing, homogenizing etc, or it may be reconstituted dried milk. An important example of "bovine milk" as described herein is pasteurized cow's milk. It is understood that the milk may be acidified, mixed or processed before, during and/or after

the inoculation with bacteria.

[0031] "Fermentation" in the methods described herein means the conversion of carbohydrates into alcohols or acids through the action of a microorganism. Preferably, fermentation in the methods of the invention comprises conversion of lactose to lactic acid.

[0032] Lactic acid bacteria are often supplied to the dairy industry either as frozen or freeze-dried cultures for bulk starter propagation or as so-called "Direct Vat Set" (DVS) cultures, intended for direct inoculation into a fermentation vessel or vat for the production of a dairy product, such as a fermented milk product. Such cultures are in general referred to as "starter cultures" or "starters".

[0033] Processes for fermentation/acidification of a milk substrate, eg for the production of cheese, are well known and the person of skill in the art will know how to select suitable process conditions, such as temperature, oxygen, amount and characteristics of microorganism(s) and process time. Obviously, fermentation conditions are selected so as to support the achievement of the present invention.

[0034] In the present context, the term "mutant" should be understood as a strain derived from a strain of the invention by means of e.g. genetic engineering, radiation and/or chemical treatment, and/or selection, adaptation, screening, etc. It is preferred that the mutant is a functionally equivalent mutant, e.g. a mutant that has substantially the same, or improved, properties (e.g. regarding yield, viscosity, gel stiffness, mouth coating, flavor, post acidification, acidification curve, acidification, acidification speed, and/or phage robustness) as the mother strain. Such a mutant is a part of the present invention. Especially, the term "mutant" refers to a strain obtained by subjecting a strain of the invention to any conventionally used mutagenization treatment including treatment with a chemical mutagen such as ethane methane sulphonate (EMS) or N-methyl-N'-nitro-N-nitroguanidine (NTG), UV light or to a spontaneously occurring mutant. A mutant may have been subjected to several mutagenization treatments (a single treatment should be understood one mutagenisation step followed by a screening/selection step), but it is presently preferred that no more than 1000, no more than 100, no more than 20, no more than 10, or no more than 5, treatments are carried out. In a presently preferred mutant, less than 5%, or less than 1% or even less than 0.1% of the nucleotides in the bacterial genome have been changed (such as by replacement, insertion, deletion or a combination thereof) compared to the mother strain. In the present context, a mutant should be of the same type as the mother strain, ie a mutant of a mother strain of type M, R or S should also be of type M, R or S, resp.

[0035] The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising", "having", "including" and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

EXAMPLES

Example 1: pH measurement using the method "Colour of pH"

MATERIALS

[0036] Transparent Nunc 96w plate (sterile)
Bromocresol purple (Aldrich)
Bromocresol green (Sigma)
Autoclaved demineralized H2O
0.2 um sterile filter
B-milk is Reconstituted Skimmed Milk (9.5%) heated to 99C for 30 min. Cf. INTERNATIONAL STANDARD ISO26323, IDF213, First edition 2009-07-01: Milk products - Determination of the acidification activity of dairy cultures by continuous pH measurement (CpH). Scanner for colour of pH (HP ScanJet 2400) pH-meter (microelectrode connected to the IQ50 pH meter (Scientific Instruments Inc., San Diego, USA))

SOLUTIONS

[0037] pH-indicator: 50 mg bromocresol purple salt (Aldrich) and 50 mg bromocresol green salt (Sigma) were dissolved in a final volume of 50 ml autoclaved demineralized H2O. The pH was adjusted to 7,0 with NaOH. The pH indicator was sterile filtered (0.2 μm) before use.
pH-indicator milk: 95 ml B-milk was mixed with 5.0 ml pH indicator

CALIBRATION

[0038] pH indicator milk was prepared with various amounts of Glucone-Delta-Lactone, in a 96 well plate, and pH was allowed to stabilize overnight at room temperature in the pH range 2.9 to 6.7. The color of the wells was measured using a flatbed scanner (HP Scanjet 2400) with appropriate scanner software (pH Multiscan v. 3.0) and pH in each well was measured with a suitable

pH meter (e.g. Radiometer's or Metrohm's), allowing correlation between the measured pH and the color (Hue) of the wells. A plot with color (Hue, horizontal axis) vs. measured pH (vertical axis) was drawn and a best fit 4th grade polynomial was used to describe the curve: pH = a*[Hue]^4 + b*[Hue]^3 + c*[Hue]^2 + d*[Hue] + e. The coefficient of correlation was greater than 0.99 (figure 1). The 4th grade polynomial was then used to calculate the pH in wells of subsequent scans.

Example 2: Assay for determination of culture type

[0039] This assay to be used to determine whether a culture is of any of type M, type S, or type R

[0040] Definition of cultures of types M, S and R:

It is understood that in the definitions the culture is grown in B-milk at 30 degrees C. In the following a "culture" refers to a pure strain (a single strain) or a co-culture of pure strains, i.e. an ensemble of pure strains grown together.

A culture of type M will have a maximum acidification rate of more than or equal to 0.05 pH units per hour but less than 0.15 pH units per hour in B-milk.

A culture of type S will have a maximum acidification rate of more than or equal to 0.40 pH units per hour in B-milk, but will not acidify B-milk added 4% salt (less than 0.05 pH units per hour).

A culture of type R will have a maximum acidification rate of more than or equal to 0.40 pH units per hour in B-milk and acidify B-milk added 4% salt (having a maximum acidification rate of more than 0.05 pH units per hour).

[0041] The pH can be measured using a conventional method, e.g. a pH electrode, or pH can be measured using Chr. Hansens proprietary method "colour of pH", see example 1 and WO2005068982A1.

[0042] Example on testing using the assay.

Materials:

[0043] M17-b: M17 (Oxoid CM 817): 37,25 g dissolved in 950 ml Millipore H2O, autoclaved for 20 min at 121 °C, and dispensed 100 ml aliquots in sterile bottles.

M17-k (M17-broth+1%glucose +1%lactose): 100 ml M17-b is supplemented with 5 ml sterile glucose stock solution (20% w/v) and 5 ml sterile lactose stock solution (20%w/v)

Salt Milk (4% sodium): 100 ml pH indicator milk was added 5.3 g NaCl.

Cultures of bacteria to be tested were grown overnight in M17k media at 30C. Strains of Lactococcus lactis were used as test cultures.

[0044] B-milk acidification: The overnight culture was added one percent to pH-indicator milk, and the pH measured every 6 minutes for 20 hours using a flatbed scanner as described in example 1.

Salt-milk acidification: The cultures were pre-incubated in B-milk and allowed to acidify for 2 hours after which they were transferred to Salt milk. pH measured by colour of pH is determined every 6th minute for 20 hrs. This protocol is part of the Primary characterization in the Strain supply chain.

Calculation of maximum acidification rates: The maximum acidification was calculated as the maximum change in pH observed between hourly pH measurements for a given pH curve, see figure 2.

Example 3: Controlling the acidification rate of defined direct to the vat cultures by blending a type M culture with a type S or type R culture

[0045] 5 g per 100 L of a type M, R or S culture produced as a defined frozen direct vat set culture was added to semi skimmed milk (1.8% fat). Then 0, 2.5, 5, 10, 20, and 30, g per 100L type M DVS culture was added.

[0046] The pH was measured at least every hour for 10 hours to obtain a pH curve using the color of pH method (example 1) (see figure 3).

[0047] When adding a type M culture to the type R and S cultures, the maximum acidification rate obtained were reduced (see figure 4). When adding type M culture to a type M culture, the maximum acidification rate was unchanged (see figure 4).

Example 4: Controlling the pH slope of blends

Materials and Methods:

[0048] A-milk is Reconstituted Skimmed Milk powder (9.5%) autoclaved at 115C for 15 min.

B-milk is Reconstituted Skimmed Milk powder (9.5%) heated to 99C for 30 min.

A suitable pH-meter giving the result with two digits (e.g. Radiometer's or Metrohm's) equipped with a pH electrode (e.g. a combination electrode from Radiometer (GK 2401C) or from Metrohm (6.0232.100)) and a thermometer for measuring the room temperature or a temperature sensor.

Experiment:

[0049] Overnight (o/n) cultures of each strain were prepared in A-milk at room temperature (20 - 24C) in 200ml sterile baby-bottles. The pH of each o/n culture was aseptically measured. The pH of the M-type strains was in the 5.5 to 5.8 range before use.

[0050] The pH of the R type strain was in the 4.5 to 4.6 range before use.

[0051] The o/n cultures were:

A) DSM 26564 in A-milk
B) DSM 26566 in A-milk
C) DSM 26569 in A-milk
D) DSM 26570 in A-milk

[0052] The o/n cultures were then combined and inoculated as follows in 200 ml B-milk in baby-bottles:

Experiment G

[0053]

G1) 1.5% (v/v) of B in B-milk (pure M type, DSM 26566)
G2) 0.1% (v/v) of A in B-milk (pure R type, DSM 26564)
G3) 1.5% (v/v) of B and 0.1% (v/v) of A in B-milk (mixed M and R type)

Experiment I

[0054]

I1) 1.5% (v/v) of D in B-milk (pure M type, DSM 26570)
I2) 0.1 % (v/v) of A in B-milk (pure R type, DSM 26564)
I3) 1.5% (v/v) of D and 0.1% (v/v) of A in B-milk (mixed M and R type)

Experiment J

[0055]

J1) 1.5% (v/v) of C in B-milk (pure M type, DSM 26569)
J2) 0.1% (v/v) of A in B-milk (pure R type, DSM 26564)
J3) 1.5% (v/v) of C and 0.1% (v/v) of A in B-milk (mixed M and R type)

[0056] The single strains and blends (experiments G, I, and J) were then fermented at 30C for 16 hours using a temperature controlled water bath. The pH was measured with a frequency of at least an hour using a suitable pH meter and electrode.

Results:

[0057] Figures 5 to 7 shows the effect of blending the same R strain with three different M strains on the slope of the acidification curve. By combining different strain in various ratios the slope of the curve can be controlled.

Example 5. Production of Camembert cheeses

Preparation of starter culture

[0058] Prepare o/n cultures A and D in A-milk from example 4 above.
[0059] Prepare a blend of A and D containing about 5% A and 95% D (Culture AD).
[0060] Centrifuge and freeze in liquid nitrogen.

First Pasteurization

[0061] Milk containing 3.74% protein and 3.5% fat is pasteurized at 73.5C for 15 seconds and cooled to about 5C

First maturation (Cold physical and biological maturation)

[0062] The milk is added 20 ml CaCl2 solution (340 g/l) per 100 liter and 8 g of the frozen culture AD per 100 liter of milk and left to ferment for 15 to 17 hours at 14.5C until pH 6.5 is reached.

Second Pasteurization

[0063] After reaching the target pH, the milk is then re-pasteurized at 73.5C for 15 seconds and cooled to 34C.

Secondary maturation

[0064] 3-5 units per 1000L PCa 1 (Penicilium candidum) and 0.5 - 1u GEO CD 1 per 1000 l (Geotrichum candidum) is added to the milk.
[0065] Finally, starter culture is added, 8 g of the frozen culture AD per 1001, and left to ferment about an hour at 34C until pH 6.30 is reached.

Renneting

[0066] At pH 6.3 about 20 ml of rennet (520 mg/l chymosin) is added to 100 liters of milk. After about 30 to 40 minutes at 34C, the now formed curd is cut into 1 cubic cm cubes.

Stirring

[0067] The cubes are gently stirred after a couple of minutes.

Molding

[0068] When pH about 6.1 - 6.2 is reached the curd is transferred into industry standard camembert molds (11 cm in diameter) and left to drain.

Draining

[0069] The cheeses are drained at different temperatures: e.g. one hour at 27C, two hours at 24C, two hours at 20C, and thereafter kept at 17C just until de-molding.
[0070] During the draining step the molds are turned twice to facilitate draining: the first time one hour after the curd was filled into molds and then again after five hours.

De-molding

[0071] After about seven hours after being filled into

molds, the cheeses are released from the molds, dry salted by hand, and transferred to the ripening room.

Ripening

[0072] The cheeses are ripened at 14 - 15°C and 85% relative humidity for one day, followed by 8 - 9 days at 10 - 13°C and 95% relative humidity. When a satisfactory surface mold growth is obtained, the cheese surface is dried and stored at 4°C.

[0073] The cheese prepared as above have an acidification profile and mineral profile as a camembert cheese produced with a undefined bulk starter, and - compared to cheese made as above, but with a single strain culture A instead of the culture AD - a higher content of calcium and a longer shelf-life.

FIGURES

[0074]

Figure 1 depicts a Graph showing measured pH (vertical axis) as a function of colour (Hue) for flat bed scanner HP2401 (monile unit). Hue values are average ± S.E. (n=4). The black line is a best fit 4th grade polynomium. The polynomium coefficients are printed on top of figure. The R2 correlation coefficient is also printed.

Figure 2 depicts examples of the acidification of lactococcus cultures of type M (M1), S (S1), and R (R2) [as measured in microtiter plates using the colour of pH method]

Figure 3 depicts the effect of adding a type M culture to type S and R cultures. 5 g culture of the S or R type per 100L was added 0, 5 and 20 g per 100L M culture. Type S cultures : SICO-14-0, SICO-O-2874, and SICO-O-S1. Type R cultures: SICO-200-O, SICO-O-R1, and SICO-OR2. Type M culture: SICO-O-M1

Figure 4 depicts the maximum acidification rate as a function of the amount of type R or S culture in the type R+M or type M+S blends

Figure 5: The effect of blending pure M (O) and pure A (□) on the shape of the acidification curve of the mix (A). See experiment G in ex 4.

Figure 6: The effect of blending pure M (O) and pure A (□) on the shape of the acidification curve of the mix (Δ). See experiment I in ex 4.

Figure 7: The effect of blending pure M (O) and pure A (□) on the shape of the acidification curve of the mix (Δ). See experiment J in ex 4.

DEPOSITS and EXPERT SOLUTION

[0075] The Applicant requests that a sample of the deposited microorganism should be made available only to an expert approved by the Applicant.

[0076] The Strains of the invention were deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstr. 7B, D-38124 Braunschweig (DSM) 31.10.2012 and given the accession numbers as follows:

Lactococcus lactis ssp. *cremoris* CHCC4421 = DSM 26561
Lactococcus lactis ssp. *lactis* CHCC9673 = DSM 26563
Lactococcus lactis ssp. *lactis* CHCC10778 = DSM 26564
Lactococcus lactis ssp. *lactis* CHCC11277 = DSM 26565
Lactococcus lactis ssp. *cremoris* CHCC11552 = DSM 26566
Lactococcus lactis ssp. *cremoris* CHCC11583 = DSM 26567
Lactococcus lactis ssp. *cremoris* CHCC11603 = DSM 26568
Lactococcus lactis ssp. *cremoris* CHCC12085 = DSM 26569
Lactococcus lactis ssp. *cremoris* CHCC13347 = DSM 26570

[0077] The deposits were made according to the Budapest treaty on the international recognition of the deposit of microorganisms for the purposes of patent procedure.

REFERENCES

[0078] B. Mieton, F. Gaucheron & F. Salaün-Michel: "Minéraux et transformations fromageres", chapter 16 in F.Gaucheron : "Minéraux et produits laitiers". Cheese and Fermented Milk Foods, by Frank V. Kosikowski. INTERNATIONAL STANDARD ISO26323 IDF213. First edition 2009-07-01 : Milk products - Determination of the acidification activity of dairy cultures by continuous pH measurement (CpH) WO2005068982A1

Claims

1. A lactic acid bacterial (LAB) culture comprising

A1) a bacterial culture of type M which is a lactic acid bacteria culture that has a maximum acidification rate of more than or equal to 0.05 pH units per hour but less than 0.15 pH units per hour in B-milk at 30 °C wherein the culture of type M comprises bacteria belonging to a strain selected from the group consisting of:

Lactococcus lactis ssp. cremoris CHCC11552 (DSM 26566);
Lactococcus lactis ssp. cremoris CHCC11583 (DSM 26567);
Lactococcus lactis ssp. cremoris CHCC11603 (DSM 26568);
Lactococcus lactis ssp. cremoris CHCC12085 (DSM 26569); and
Lactococcus lactis ssp. cremoris CHCC13347 (DSM 26570);
and mutants thereof,

A2) a bacterial culture of type R which is a lactic acid bacteria culture that has a maximum acidification rate of more than or equal to 0.40 pH units per hour in B-milk at 30 °C, and has a maximum acidification rate of less than 0.05 pH units per hour in B-milk added 4% salt at 30 °C; and
A3) optionally a bacterial culture of type S which is a lactic acid bacteria culture that has a maximum acidification rate of more than or equal to 0.40 pH units per hour in B-milk at 30 °C and has a maximum acidification rate of more than 0.05 pH units per hour in B-milk added 4% salt at 30 °C, wherein the culture of type R comprises bacteria belonging to a strain selected from the group consisting of:

Lactococcus lactis ssp. lactis CHCC10778 (DSM 26564); and
Lactococcus lactis ssp. lactis CHCC11277 (DSM 26565);
and mutants thereof and wherein, the culture of type S comprises bacteria belonging to a strain selected from the group consisting of:

Lactococcus lactis ssp. cremoris CHCC4421 (DSM 26561); and
Lactococcus lactis ssp. lactis CHCC9673 (DSM 26563);
and mutants thereof.

2. A culture of the preceding claim comprising

A1) a bacterial culture of type M; and
A2) a bacterial culture of type R; and
A3) a bacterial culture of type S.

3. The culture of any preceding claim, which comprises

A1) 5-90% of the culture of type M; and
A2) 5-90% of the culture of type R; and
A3) 0-90% of the culture of type S;

the ratio is calculated as CFU relative to the total CFU of the culture.

4. The culture of any preceding claim, which comprises

A1) 60-98% of the culture of type M; and
A2) 1-20% of the culture of type R; and
A3) 1-20% of the culture of type S;

the ratio is calculated as CFU relative to the total CFU of the culture.

5. The culture of any preceding claim, which comprises

A1) 5-90% of the culture of type M; and
A2) 5-90% of the culture of type R; and
A3) 5-90% of the culture of type S;

the ratio is calculated as CFU relative to the total CFU of the culture.

6. The culture of any preceding claim, which comprises from 10E8 to 10E12 CFU of the culture of type M per g culture.

7. The culture of any preceding claim, which comprises from 10E8 to 10E12 CFU of the culture of type R per g culture.

8. The culture of any preceding claim, which comprises from 10E8 to 10E12 CFU of the culture of type S per g culture.

9. The culture of any preceding claim, which is in liquid, frozen or freeze-dried form.

Patentansprüche

1. Milchsäurebakterienkultur (lactic acid bacterial culture - LAB-Kultur), umfassend:

A1) eine Bakterienkultur des Typs M, bei der es sich um eine Milchsäurebakterienkultur handelt, die ein maximales Versauerungsverhältnis von größer oder gleich 0,05 pH-Einheiten pro Stunde, jedoch kleiner 0,15 pH-Einheiten pro Stunde in B-Milch bei 30°C aufweist, wobei die Kultur des Typs M Bakterien umfasst, die einem Stamm angehören, der aus der Gruppe ausgewählt ist, die aus Folgendem besteht:

Lactococcus lactis ssp. cremoris CHCC11552 (DSM 26566);
Lactococcus lactis ssp. cremoris CHCC11583 (DSM 26567);
Lactococcus lactis ssp. cremoris CHCC11603 (DSM 26568);
Lactococcus lactis ssp. cremoris CHCC12085 (DSM 26569); und
Lactococcus lactis ssp. cremoris

CHCC13347 (DSM 26570);
und Mutationen davon,

A2) eine Bakterienkultur des Typs R, bei der es sich um eine Milchsäurebakterienkultur handelt, die ein maximales Versauerungsverhältnis von größer oder gleich 0,40 pH-Einheiten pro Stunde in B-Milch bei 30°C aufweist und ein maximales
Versauerungsverhältnis von kleiner 0,05 pH-Einheiten in B-Milch mit 4 % hinzugefügtem Salz bei 30°C aufweist; und
A3) gegebenenfalls eine Bakterienkultur des Typs S, bei der es sich um eine Milchsäurebakterienkultur handelt, die eine maximale Versauerungsrate von größer oder gleich 0,40 pH-Einheiten pro Stunde in B-Milch bei 30°C aufweist und eine maximale Versauerungsrate von größer 0,05 pH-Einheiten pro Stunde in B-Milch mit 4 % hinzugefügtem Salz bei 30°C aufweist, wobei die Kultur des Typs R Bakterien umfasst, die einem Stamm angehören, der aus der Gruppe ausgewählt ist, die Folgendes umfasst:

Lactococcus lactis ssp. lactis CHCC10778 (DSM 26564); und
Lactococcus lactis ssp. lactis CHCC11277 (DSM 26565) ;
und Mutationen davon und wobei die Kultur des Typs S Bakterien umfasst, die einem Stamm angehören, der aus der Gruppe ausgewählt ist, die Folgendes umfasst:

Lactococcus lactis ssp. Cremoris CHCC4421 (DSM 26561); und
Lactococcus lactis ssp. lactis CHCC9673 (DSM 26563); und Mutationen davon.

2. Kultur nach dem vorangehenden Anspruch, umfassend:

A1) eine Bakterienkultur des Typs M; und
A2) eine Bakterienkultur des Typs R; und
A3) eine Bakterienkultur des Typs S.

3. Kultur nach einem der vorangehenden Ansprüche, die Folgendes umfasst:

A1) 5-90 % der Kultur des Typs M; und
A2) 5-90 % der Kultur des Typs R; und
A3) 0-90 % der Kultur des Typs S;

wobei das Verhältnis als CFU bezogen auf die gesamte CFU der Kultur berechnet wird.

4. Kultur nach einem der vorangehenden Ansprüche, die Folgendes umfasst:

A1) 60-98 % der Kultur des Typs M; und
A2) 1-20 % der Kultur des Typs R; und
A3) 1-20 % der Kultur des Typs S;

wobei das Verhältnis als CFU bezogen auf die gesamte CFU der Kultur berechnet wird.

5. Kultur nach einem der vorangehenden Ansprüche, die Folgendes umfasst:

A1) 5-90% der Kultur des Typs M; und
A2) 5-90% der Kultur des Typs R; und
A3) 5-90 % der Kultur des Typs S;

wobei das Verhältnis als CFU bezogen auf die gesamte CFU der Kultur berechnet wird.

6. Kultur nach einem der vorangehenden Ansprüche, die zwischen 10E8 und 10E12 CFU der Kultur des Typs M pro g Kultur umfasst.

7. Kultur nach einem der vorangehenden Ansprüche, die zwischen 10E8 und 10E12 CFU der Kultur des Typs R pro g Kultur umfasst.

8. Kultur nach einem der vorangehenden Ansprüche, die zwischen 10E8 und 10E12 CFU der Kultur des Typs S pro g Kultur umfasst.

9. Kultur nach einem der vorangehenden Ansprüche, die in flüssiger, gefrorener oder gefriergetrockneter Form vorliegt.

Revendications

1. Culture bactérienne lactique (LAB) comprenant

A1) une culture bactérienne de type M qui est une culture de bactéries lactiques qui présente un taux d'acidification maximal supérieur ou égal à 0,05 unité pH par heure, mais inférieur à 0,15 unité pH par heure dans du lait B à 30 °C, dans laquelle la culture de type M comprend des bactéries appartenant à une souche sélectionnée à partir du groupe constitué de :

Lactococcus lactis ssp. *cremoris* CHCC11552 (DSM 26566) ;
Lactococcus lactis ssp. *cremoris* CHCC11583 (DSM 26567) ;
Lactococcus lactis ssp. *cremoris* CHCC11603 (DSM 26568) ;
Lactococcus lactis ssp. *cremoris* CHCC12085 (DSM 26569) ; et
Lactococcus lactis ssp. *cremoris* CHCC13347 (DSM 26570) ;
et des mutants de celles-ci,

A2) une culture bactérienne de type R qui est une culture de bactéries lactiques qui présente un taux d'acidification maximal supérieur ou égal à 0,40 unité pH par heure dans du lait B à 30 °C, et présente un taux d'acidification maximal inférieur à 0,05 unité pH par heure dans du lait B complémenté de 4 % de sel à 30 °C ; et
A3) éventuellement une culture bactérienne de type S qui est une culture de bactéries lactiques qui présente un taux d'acidification maximal supérieur ou égal à 0,40 unité pH par heure dans du lait B à 30 °C et présente un taux d'acidification maximal supérieur à 0,05 unité pH par heure dans du lait B additionné de 4 % de sel à 30 °C,
dans laquelle la culture de type R comprend des bactéries appartenant à une souche sélectionnée à partir du groupe constitué de :

Lactococcus lactis ssp. *lactis* CHCC10778 (DSM 26564) ; et
Lactococcus lactis ssp. *lactis* CHCC11277 (DSM 26565) ;
et des mutants de celles-ci et dans laquelle la culture de type S comprend des bactéries appartenant à une souche sélectionnée à partir du groupe constitué de :

Lactococcus lactis ssp. *cremoris* CHCC4421 (DSM 26561) ; et
Lactococcus lactis ssp. *lactis* CHCC9673 (DSM 26563) ;
et des mutants de celles-ci.

2. Culture selon la revendication précédente comprenant

A1) une culture bactérienne de type M ; et
A2) une culture bactérienne de type R ; et
A3) une culture bactérienne de type S.

3. Culture selon l'une quelconque des revendications précédentes, qui comprend

A1) 5 à 90 % de la culture de type M ; et
A2) 5 à 90 % de la culture de type R ; et
A3) 0 à 90 % de la culture de type S ;

le rapport est calculé en UFC par rapport à l'UFC totale de la culture.

4. Culture selon l'une quelconque des revendications précédentes, qui comprend

A1) 60 à 98 % de la culture de type M ; et
A2) 1 à 20 % de la culture de type R ; et
A3) 1 à 20 % de la culture de type S ;

le rapport est calculé en UFC par rapport à l'UFC totale de la culture.

5. Culture selon l'une quelconque des revendications précédentes, qui comprend

A1) 5 à 90 % de la culture de type M ; et
A2) 5 à 90 % de la culture de type R ; et
A3) 5 à 90 % de la culture de type S ;

le rapport est calculé en UFC par rapport à l'UFC totale de la culture.

6. Culture selon l'une quelconque des revendications précédentes, qui comprend de 10E8 à 10E12 UFC de la culture de type M par g de culture.

7. Culture selon l'une quelconque des revendications précédentes, qui comprend de 10E8 à 10E12 UFC de la culture de type R par g de culture.

8. Culture selon l'une quelconque des revendications précédentes, qui comprend de 10E8 à 10E12 UFC de la culture de type S par g de culture.

9. Culture selon l'une quelconque des revendications précédentes, qui est sous forme liquide, congelée ou lyophilisée.

FIG 1

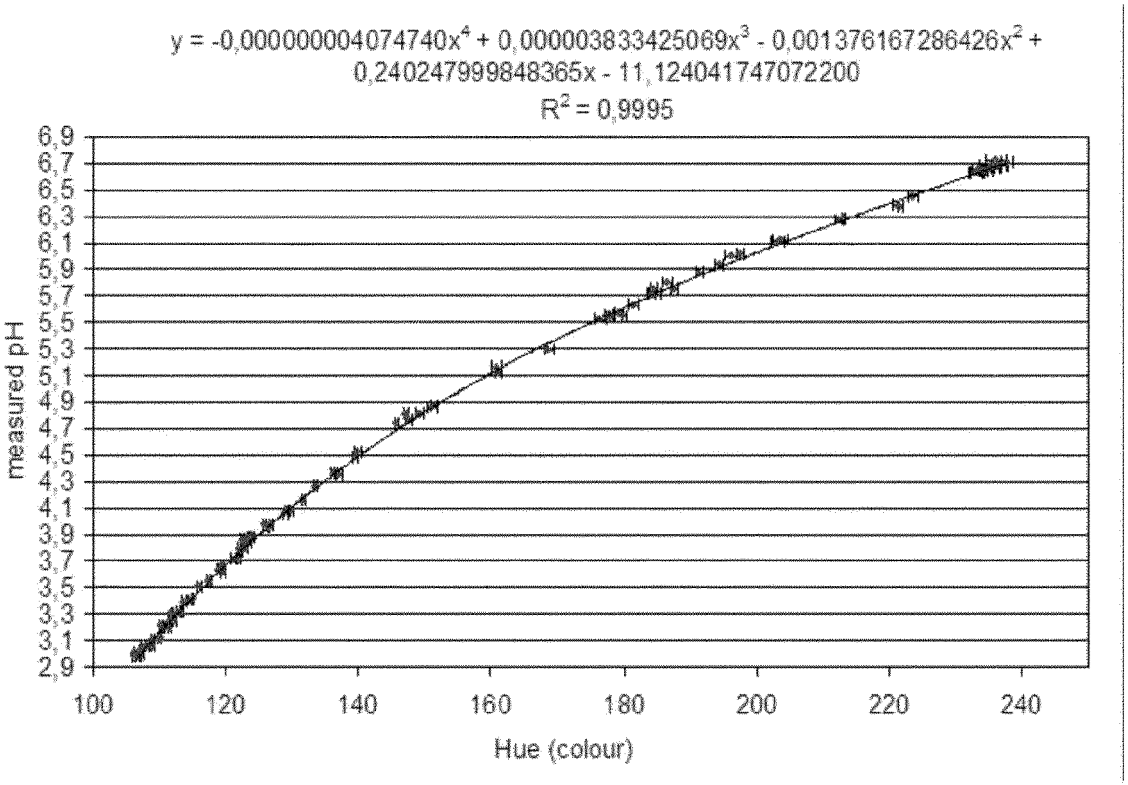
y = -0,000000004074740x⁴ + 0,000003833425069x³ - 0,001376167286426x² + 0,240247999848365x - 11,124041747072200
R² = 0,9995
measured pH
6,9
6,7
6,5
6,3
6,1
5,9
5,7
5,5
5,3
5,1
4,9
4,7
4,5
4,3
4,1
3,9
3,7
3,5
3,3
3,1
2,9
100
120
140
160
180
200
220
240
Hue (colour)

FIG 2

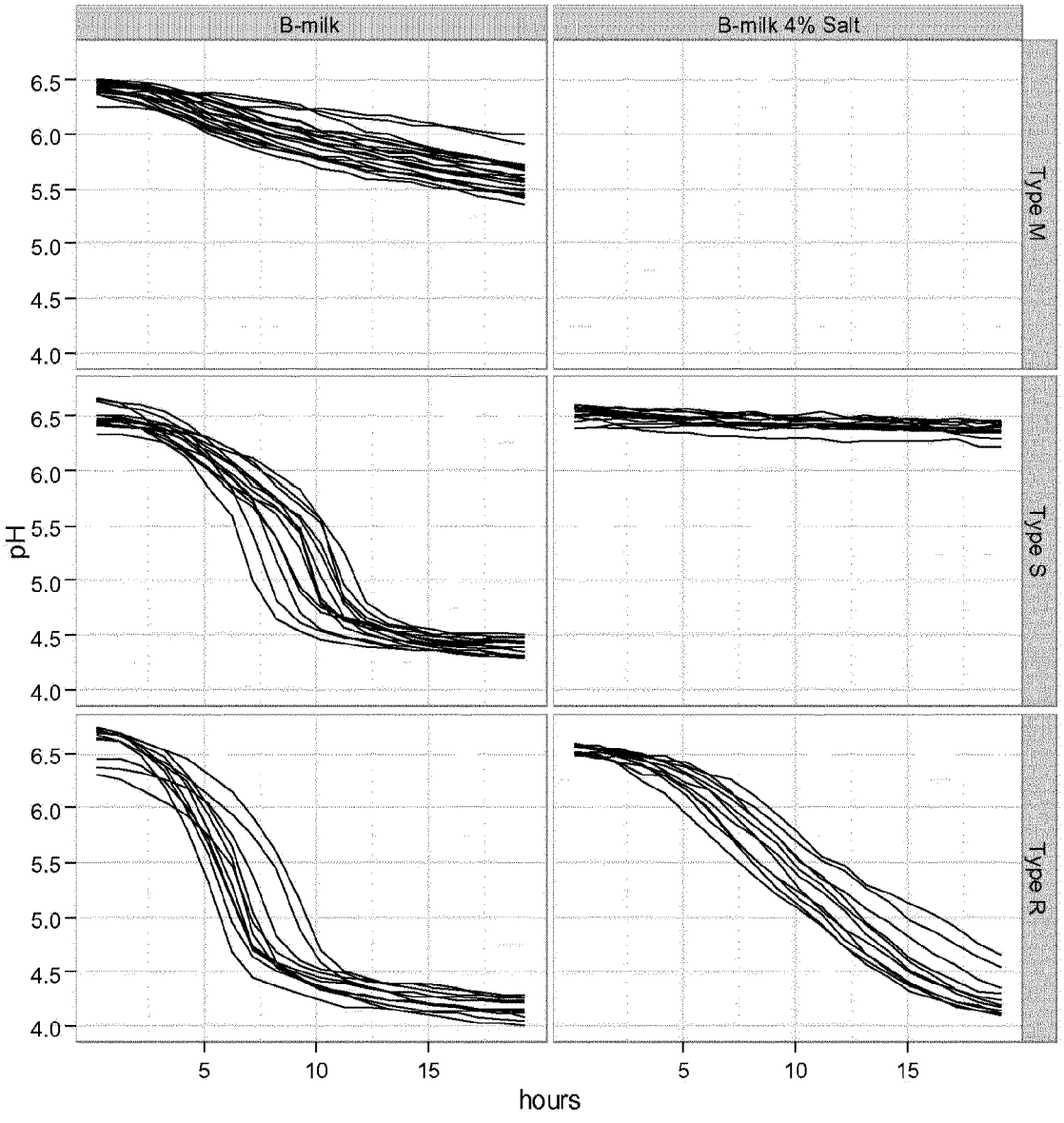
B-milk
B-milk 4% Salt
Type M
Type S
Type R
pH
6.5
6.0
5.5
5.0
4.5
4.0
5
10
15
hours

FIG 3

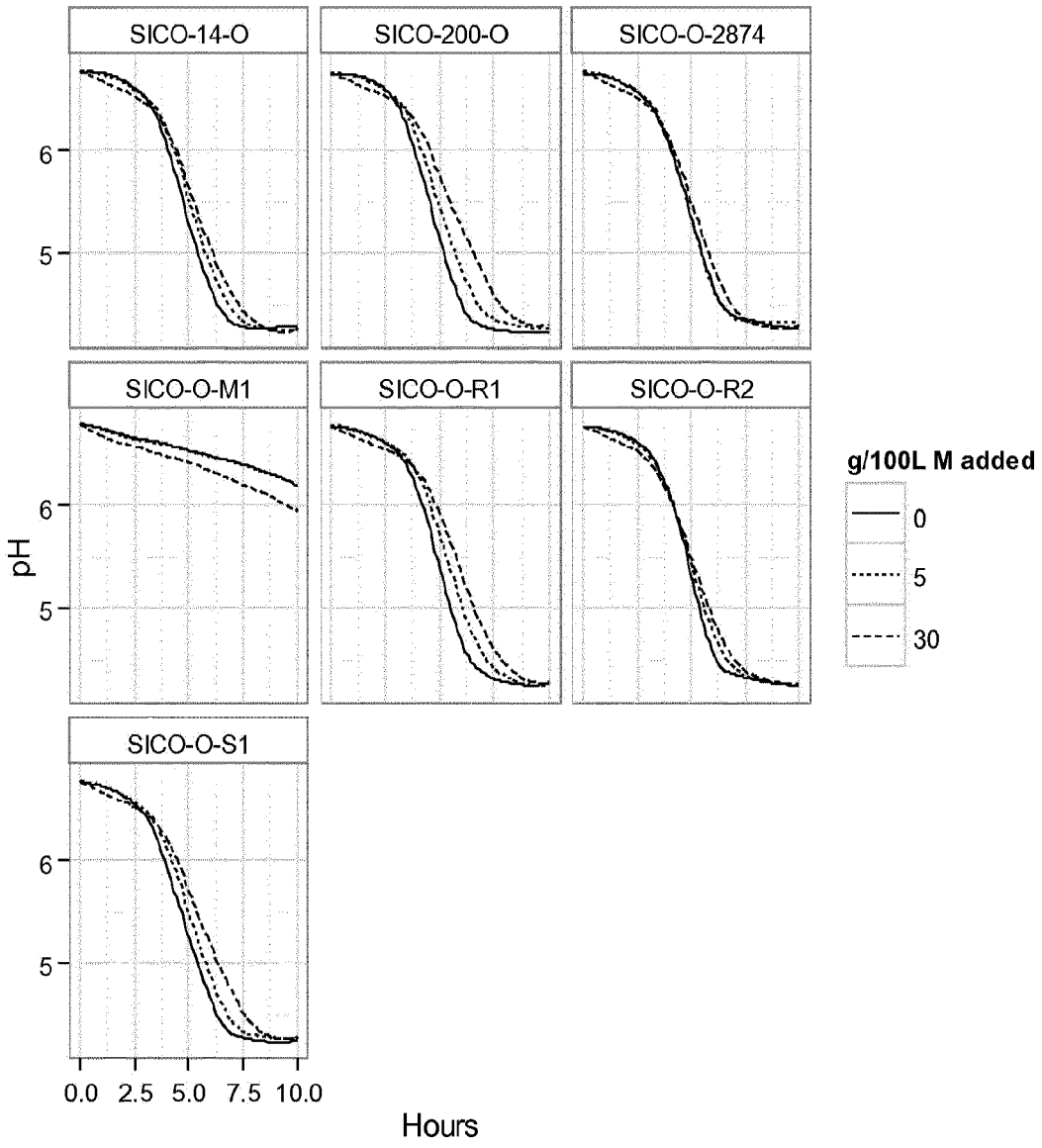
SICO-14-O
SICO-200-O
SICO-O-2874
SICO-O-M1
SICO-O-R1
SICO-O-R2
SICO-O-S1
pH
6
5
0.0
2.5
5.0
7.5
10.0
Hours
g/100L M added
0
5
30

FIG 4

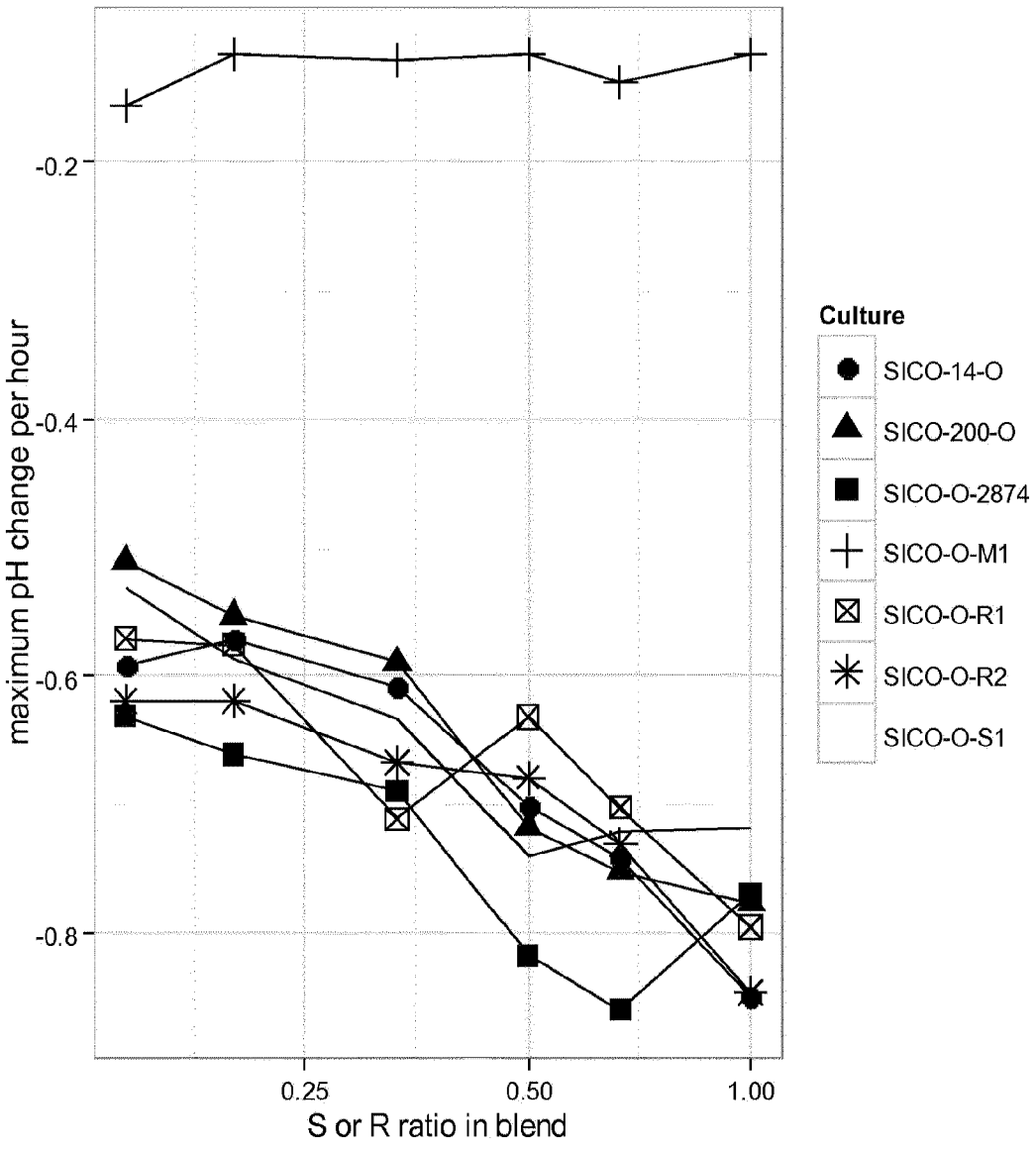
maximum pH change per hour
-0.2
-0.4
-0.6
-0.8
0.25
0.50
1.00
S or R ratio in blend
Culture
SICO-14-O
SICO-200-O
SICO-O-2874
SICO-O-M1
SICO-O-R1
SICO-O-R2
SICO-O-S1

FIG 5

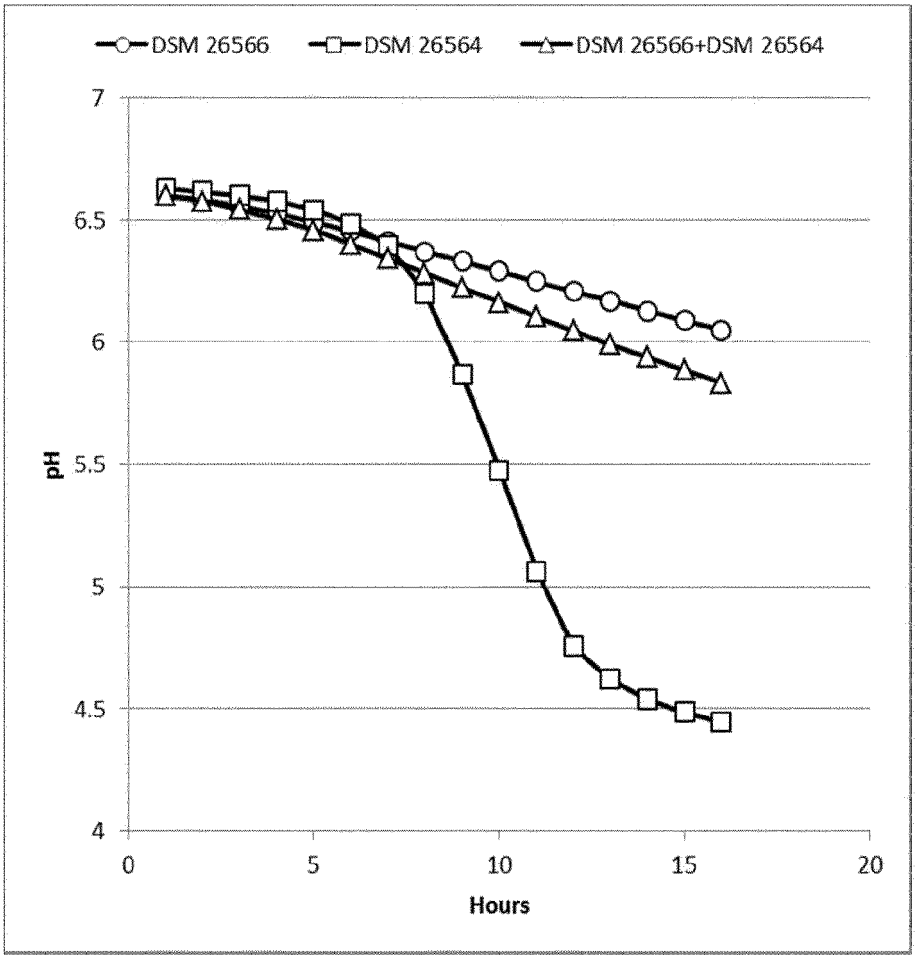

Figure 5: experiment G. The effect of blending pure M (○) and pure A (□) on the shape of the acidification curve of the mix (Δ).

FIG 6

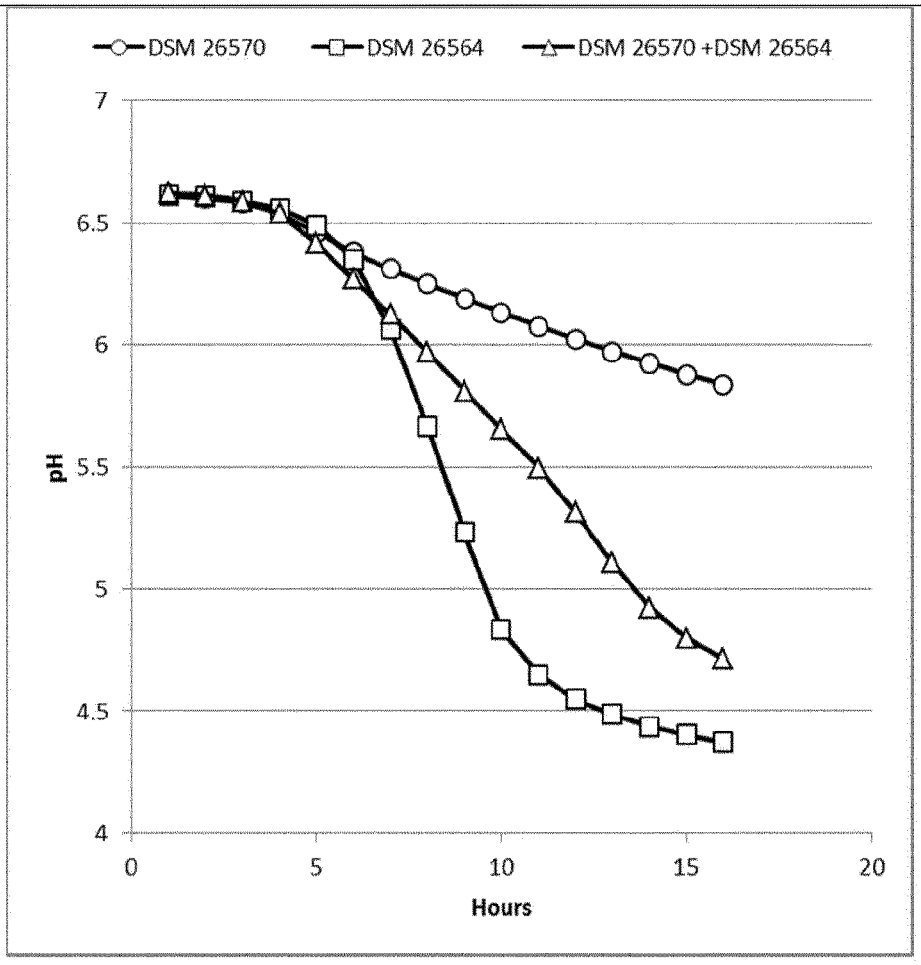

Figure 6: experiment I. The effect of blending pure M (○) and pure A (□) on the shape of the acidification curve of the mix (Δ).

FIG 7

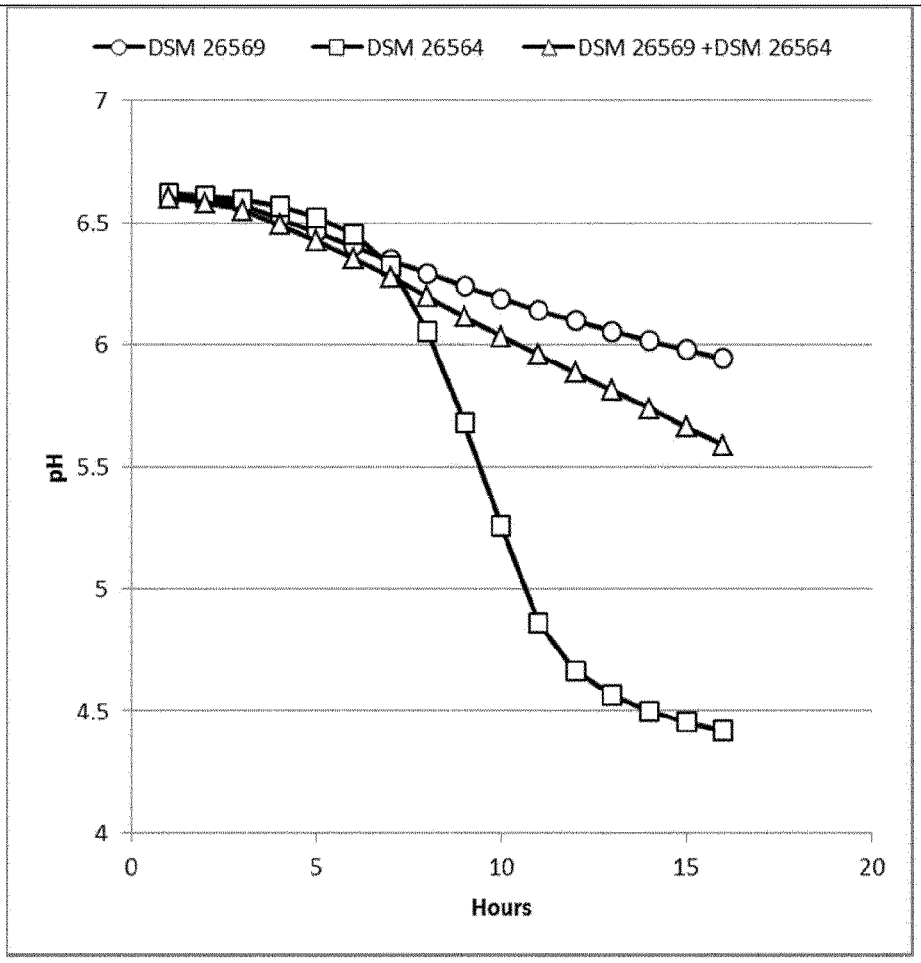

Figure 7: experiment J. The effect of blending pure M (○) and pure A (□) on the shape of the acidification curve of the mix (Δ).

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- WO 2005068982 A1 **[0041] [0078]**

Non-patent literature cited in the description

- **BARRETTE J et al.** *JOURNAL OF INDUSTRIAL MICROBIOLOGY AND BIOTECHNOLOGY, BASINGSTOKE, GB,* 01 December 2000, vol. 25 (6), ISSN 1367-5435, 288-297 **[0008]**